against hIL-3 as well as with antibodies raised against the predicted *B. subtilis* SsrA-tag (Fig. 4, lane 3 and 8). This band is absent in the medium of *B. subtilis* 168 (pLATIL3TERM) (lanes 2 and 7) and WB600 Δ*ssrA* (pLATIL3TERM) (lanes 4 and 9). Thus, hIL-3 molecules translated from mRNAs that lack termination codons are tagged by *B. subtilis* SsrA. The fact that these tagged molecules react with antibodies raised against the predicted *B. subtilis* SsrA peptide tag (AGKTNSFNQNVALAA) indicates that this prediction, which was based on comparative sequence analysis of SsrA sequences of several bacteria (Williams 2000), was correct. In addition, it can be concluded that at least one of the major extracellular proteases of *B. subtilis* (those that are absent in WB600) plays a role in the degradation of extracellular, SsrA-tagged h-IL3. When SsrA is absent, stalled ribosomes are released by an SsrA-independent mechanism, referred to as 'run-off translation' (Williams et al. 1999. Resuming translation on tmRNA: a unique mode of determining a reading frame. EMBO J. 18:5423-5433). The upper band in lane 4 probably represents the run-off translation product of full-length hIL-3 mRNA from pLATIL3TERM, while the bands with lower molecular weight are most likely degradations products thereof. It seems that some run-off translation product is also formed when SsrA is present (lane 3), but it cannot be excluded that this band is just an N-terminal degradation product of SsrA-tagged hIL-3.

As a second approach to detect SsrA-tagged proteins, we constructed *B. subtilis* strains that express an SsrA variant ($SsrA^{DD}$), in which the final two codons of the peptide reading frame are changed to encode aspartic acid residues instead of alanines. As mentioned above, it was shown in *E. coli* that an $SsrA^{DD}$ variant mediates the addition of a peptide tag that does not lead to rapid degradation (Abo et al. 2000; Karzai et al. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J. 18:3793-3799). Evaluation of the antibodies that were raised against the predicted *B. subtilis* SsrA tag (AGKTNSFNQNVALAA)

showed that they recognize the hIL-3 fused at the C-terminus to either the wild-type tag (AA-tag) or the protease resistant DD-tag (AGKTNSFNQNVALDD) (data not shown). Human IL-3 molecules tagged by SsrA^{DD} and subsequently secreted (Fig 4, lanes 5 and 10) are indeed relatively more stable then hIL-3 molecules tagged by wild-type SsrA (lanes 3 and 8), even in the six-fold protease negative strain WB600. The level of full-length SsrA^{DD}-tagged hIL-3 in the medium is somewhat higher then that of (wild-type) SsrA-tagged h-IL3 (Fig. 3, compare lane 10 with lane 8) and relatively few degradation products of SsrA^{DD}-tagged hIL-3 were detected with anti-hIL-3 antibody (compare lane 5 with lane 3). This observation suggests that besides the major extracellular proteases that are deleted in WB600, one (or more) additional protease is involved in the degradation of SsrA-tagged hIL-3. Therefore, we studied the role of three other proteases with respect to degradation of SsrA-tagged hIL-3.

CtpA has an additional role in the degradation of SsrA-tagged hIL-3 secreted by *B. subtilis*. Three derivatives of WB600 were constructed. One WB600 Δ*ctpA*, carried a deletion in the *ctpA* gene, a homologue of the *E. coli* gene encoding Tsp (tail specific protease). The other two, WB600 Δ*yvjB* and WB600 I*clpP*, carried a deletion of the *yvjB* gene (also a homologue of *E. coli tsp*) or the *clpP* gene placed under control of the IPTG-dependent P*spac* promoter of pMutin2, respectively. These three strains, together with WB600 and WB600 Δ*ssrA*, were transformed with plasmid pLATIL3TERM, grown in TSB medium with neomycin, and culture supernatants of cells entering the stationary phase were analyzed by Western blotting with anti-hIL-3 antibodies and anti-Bs-SsrAtag antibodies (Fig 5). SsrA-tagged h-IL3 could not be detected in the medium of cells lacking SsrA (Fig. 5, lanes 5 and 10), but was present when cells contained functional SsrA (all other lanes). As observed previously (Fig. 3), it seems that some full-length, run-off translation product is not only formed when cells lack SsrA (Fig. 5, lane 5), but also when SsrA is present (lanes 1-4). However, as

mentioned before, it cannot be excluded that the protein bands in lanes 1-4, which have the same mobility as full-length, run-off product (upper band in lane 5), represent a degradation product of SsrA-tagged hIL-3. Inactivation of *yvjB* or *clpP* in WB600 did not alter the amount of SsrA-tagged hIL-3 in the medium (compare lanes 1 and 6 with lanes 3 and 8, and lanes 4 and 9). The absence of functional CtpA in WB600, however, leads to a higher amount of SsrA-tagged hIL-3 in the medium (lanes 2 and 7) and also to a lower amount of the two smallest degradation products of hIL-3 (lane 2). Thus, the protease CtpA also plays a role in the degradation of SsrA-tagged h-IL3 secreted by *B. subtilis*.

Example 5

SsrA tagging of native B. subtilis proteins

B. subtilis 168 IssrA^{DD} expressing the variant SsrA^{DD} RNA, containing the protease-resistant DD-tag sequence, was analyzed by Western blotting using the anti-Bs-SsrAtag antibodies to detect native proteins of B. subtilis that are tagged through the SsrA system. As controls, cells of B. subtilis 168 (expressing wild-type SsrA) and 168 ΔssrA were used. Samples were taken of cells that were in the exponential growth phase or in the stationary phase, and the intracellular proteins and the extracellular proteins were analyzed separately. A large number of intracellular proteins were detected by anti-Bs-SsrAtag antibody when cells expressed SsrA^{DD} (Fig 6, lanes 2 and 8), while almost all of these bands were absent in cells expressing either wild-type SsrA (lanes 1 and 7) or no SsrA (lanes 3 and 9). As observed in E. coli (Abo et al. 2000), these results indicate that many endogenous cellular proteins were tagged by the SsrA system, resulting in chimeric proteins. The proteins with the wild-type SsrA tag (AA-tag) are subsequently degraded by proteases, while proteins with the DD-tag escape from proteolysis. While in the exponential growth phase the majority of the reacting bands were of relatively low molecular weight (lane 2), in the stationary phase a shift was observed